RADIAL IMMUNODIFFUSION

This protocol is based on the EDVOTEK® protocol "Radial Immunodiffusion".

10 groups of students

1. EXPERIMENT OBJECTIVE

Radial Immunodiffusion is a sensitive quantitative technique that is often used clinically to detect patient levels of blood proteins. In this experiment, students will learn to quantitatively determine the unknown concentration of an antigen.

2. EXPERIMENT COMPONENTS for 10 groups of students

COMPONENTS	Store
A. Antibody solution	4-8ºC
B. Standard antigen solution	4-8ºC
C. UltraSpec-Agarose™	4-8ºC
D. Buffer powder	4-8ºC
E. Unknown concentration of antigen	4-8ºC
1 Sleeve Petri dishes	
2 10 ml pipettes	
10 Well cutters	
80 Transfer pipettes	
70 Microtest tubes	
1 Graph paper templates	
1 Practice loading solution	

NOTE: Store all components at the indicated temperatures upon receipt.

NOTE: No human material is used in this practice.

NOTE: All components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

2.1 Requirements

- Automatic Micropipets and Tips (5-50 µl)
- Pipet Pumps (for 10 ml pipets)
- Ruler
- Plastic Box or Dish
- Plastic Wrap
- Foil
- Paper Towels
- Distilled Water
- Heat plate, Bunsen burner, or microwave
- 400 to 600 ml beaker or Erlenmeyer flask

- 150 ml beaker or flask
- Water bath
- 250 ml Graduated Cylinder
- 37°C Incubation Oven

NOTE: Make sure glassware is clean, dry, and free of soap residue. For convenience, you can buy additional disposable transfer pipettes to the steps of extraction and washing liquids.

3. BACKGROUND INFORMATION

Radial immunodiffusion

The fundamental reaction of immunology involves the interaction of **antibodies (Ab)** and **antigens (Ag)**. These interactions are useful in the defense of the body against bacterial and viral infections and toxins. The defense capabilities are dependent upon the recognition of antigens by humoral components of the immune system. Specific antibodies are then produced in response to exposure to the antigen.

The formation of **antigen-antibody complexes** is the first step in removing infectious agents from the body. Because each antibody can bind more than one antigen and each antigen can be bound by more than one antibody molecule, very large macromolecular complexes can form. These complexes form precipitates which can be cleared from the body through various means. These precipitates are also useful for laboratory and diagnostic tests.

When antibodies and antigens are inserted into different areas of an agarose gel, they diffuse toward each other and form opaque bands of precipitate at the interface of their diffusion fronts. Precipitation reactions of antibodies and antigens in agarose gels provide a method of analyzing the various antibody-antigen reactions in a system.

Double diffusion in two dimensions is a simple procedure invented by the Swedish scientist, Ouchterlony. Antigen and antibody solutions are placed in separate wells cut in an agarose plate. The reactants diffuse from the wells toward each other and precipitate where they meet at equivalent proportions. A single antigen will combine with its homologous antibody to form a single precipitation line.

Radial immunodiffusion (RID) is a technique that can quantitatively deter- mine the concentration of an antigen. Unlike many gel and liquid precipitation techniques which qualitatively detect antigen, RID is a sensitive quantitative technique that is often used clinically to detect patient levels of blood proteins.

Antibody is incorporated into molten agarose which is poured into a Petri dish and allowed to solidify. Small wells are cut into the agarose and are filled with known concentrations of antigen which corresponds to the antibody in the agarose. Samples of unknown concentrations are placed in similar wells. The antigens in solution then diffuse outwards from the well in a circular pattern surrounding the well. Antibody is present in excess and diffusion of the antigen will continue until a stable ring of antigen-antibody precipitate forms. There are antigen-antibody complexes throughout the zone surrounding the well within the precipitin line. At the precipitin line is where the greatest number of complexes can be found because the antigen and antibody are present in roughly equal proportions. This is known as the **equivalence zone** or **equivalence point**. Generally, it takes 24 to 48 hours for optimal diffusion to occur and precipitation to become apparent.

For each antigen, an endpoint precipitation ring of a certain diameter will form. From the known standard concentrations, a standard curve can be drawn by plotting antigen concentration versus the diameter squared measurements of the rings. From this linear calibration curve the concentration of the unknown antigen samples may be determined.

4. EXPERIMENTAL PROCEDURES

Radial Immunodiffusion is a sensitive quantitative technique that is often used clinically to detect patient levels of blood proteins. In this experiment, students will learn to quantitatively determine the unknown concentration of an antigen.

4.1 Laboratory safety

No human material is used in this experiment.

1. Gloves and safety goggles should be worn at all times as good laboratory practice.

2. NOT PIPETTE WITH THE MOUTH, use appropriate devices.

3. Exercise caution when working with equipment using together heat and mix of reagents.

4. Wash hands with soap and water after working in the laboratory or after using biological reagents and materials.

5. Be careful when using any electrical equipment in the laboratory.

6. Dispose of RID plates using appropriate laboratory waste disposal procedures.

If you are unsure of something, ASK YOUR INSTRUCTOR

4.2 Approximate time requirements for pre-lab and experimental procedures

Your individual schedule and time requirements will determine when the RID plates should be prepared. It takes approximately 30 minutes to prepare the plates (generally 10 minutes of this time is required for solidification).

Students can prepare the plates, if time allows.

4.3 PreLab Preparations

Notes preparations teacher practice

The class size, length of classes of practices and equipment availability are factors that must be considered in the planning and implementation of this practice with their students. These guidelines can be adapted to fit your specific circumstances.

Laboratory notebooks:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Registration laboratory activities

Students must register in their book practices the activities listed below.

Before starting the experiment:

- Write a hypothesis that reflects the experiment.
- Predict experimental outcomes.

During the Experiment:

- Record (draw) your observations, or photograph the results.

Following the Experiment:

- Formulate an explanation from the results.
- Determine what could be changed in the experiment if the experiment were repeated.
- Write a hypothesis that would reflect this change.

General instructions
A. PREPARATIONS BEFORE PRACTICE
PREPARE AGAROSE IN BUFFER

1. In a 400 to 600 ml beaker or Erlenmeyer flask, add entire contents of buffer powder package (component D) to 200 ml distilled water. Swirl the flask until the powder is in solution. Remove 50 ml for use as dilution buffer to a separate beaker.
2. Add the entire contents of agarose package (component C) to the flask or beaker containing 150 ml of buffer. Swirl to disperse large clumps. With a marking pen, indicate the level of solution volume on the outside of the flask or beaker.
3. The solution must be boiled to dissolve the agarose. This can be accomplished with a hot plate or microwave. Cover the beaker with foil and heat the mixture to boiling over the burner with occasional swirling. Wear safety goggles and use hot gloves. Boil until all the agarose is dissolved. Check to make sure that there are no small, clear particles of agarose. The final solution should be clear.

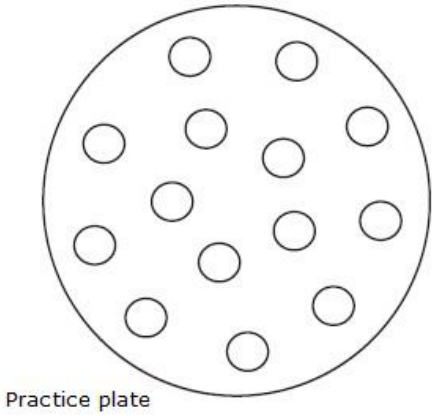
   Heat the mixture to dissolve the agarose powder. The final solution should be clear (like water) without any undissolved particles.

   A. Microwave method:
      - Cover flask with plastic wrap to minimize evaporation.
      - Heat the mixture on High for 1 minute.
      - Swirl the mixture and heat on High in bursts of 25 seconds until all the agarose is completely dissolved.

   B. Hot plate method:
      - Cover the flask with foil to prevent excess evaporation.
      - Heat the mixture to boiling over a burner with occasional swirling. Boil until all the agarose is completely dissolved.
4. If detectable evaporation has occurred, add hot distilled water to adjust the volume of solution up to the original level as marked on the flask or beaker in step 2. Do not use cool water, or the agarose solution may cool too quickly and prematurely solidify.
5. Cool the agarose solution to 55°C in a waterbath. Swirl occasionally while cooling.

PREPARATION OF PRACTICE PLATES

1. If practice plates are to be made, pipette 2.5 ml of molten agarose into each of 10 petri dishes with a 10 ml pipette. Gently spread the agarose with the pipette on the bottom of the plate to cover the entire surface. Return the remaining agarose to waterbath.
2. Allow agarose plates to set up and cool. Refrigerate if plates are not to be used within a few hours.



Practice plate

PREPARATION OF ANTIBODY PLATES

1. Pour 26 ml of molten agarose solution to a large tube or flask.
2. Add entire contents of Antibody Solution (Component A) to the 26 ml warm agarose solution. With a pipet, stir the solution to mix. Keep the solution warm (such as in the 55°C waterbath) so it does not prematurely set up. The antibody concentration will be 1 mg/ml.

NOTE: Ensure that the temperature of the agarose solution is at 55°C by adding the Antibody Solution to prevent degradation of the antibodies.

3. With a 10 ml pipette, dispense 2.5 ml into the bottom of each Petri dish, gently spread the agarose with the pipette to cover the bottom. Allow the agarose to solidify. This will take approximately 10 minutes. If the plates are not to be used the day of the preparation, they can be wrapped in plastic wrap and stored in the refrigerator for no longer than one week.
4. Each group requires 1 antibody plate and 1 practice plate.

B. PREPARATIONS ON THE PRACTICE DAY
PREPARATION OF ANTIGENS

Students will prepare serial dilutions of the Standard Antigen Solution (Component B) to determine the standard curve.

1. Label 10 microtest tubes with "Standard".
2. Label 10 microtest tubes with "Unknown".
3. Label 10 microtest tubes with "Buffer".
4. Aliquot 75 µl of Standard Antigen Solution (Component B) into each tube labeled "Standard".
5. Aliquot 10 µl of Unknown Antigen Solution (Component E) into each tube labeled "Unknown".
6. Aliquot 1 ml of Buffer (retained from plate preparation step) into each tube labeled "Buffer".
7. Each group requires one tube each of Standard, Unknown, and Buffer.

NOTE: Solutions can be aliquoted before the day of practice, in which case they should be stored at 4-8°C until the day of practice.

PREPARATION OF INCUBATION CHAMBER

1. Obtain plastic container or dish with lid. If a lid is not available, the container may be covered with plastic wrap.

2. Line the bottom of the container with several paper towels. Add distilled water to the towels to saturate. There should not be any liquid above the paper towels. All the liquid should be absorbed into the towels. Cover the chamber with the lid or plastic wrap.

4.4 Material that should receive each group

Distribute the following to each student group, or set up a work station for students to share materials.

- 1 tube Buffer
- 1 tube Standard
- 1 tube Unknown
- 4 microtest tubes
- 1 practice plate
- 1 experimental RID plate
- 1 well cutter
- 1 template
- Micropipetting device and tips (or 8 transfer pipets)
- Graph paper
- Ruler
- Marking pen

4.5 Avoiding common pitfalls

1. Follow instructions carefully when preparing gels. Make sure the agarose is completely dissolved.
2. Make neat, clean wells with the well cutters. Take measures to ensure that the wells are properly spaced according to the template on page 5.
3. Add samples to the wells carefully and precisely. Avoid overfilling the wells.
4. Do not tip or invert plates when transferring to the humidity chamber.
5. Placing the humidity chamber in a 37°C incubation oven will expedite the formation of precipitin arcs.

5. STUDENT EXPERIMENTAL PROCEDURES

A. PREPARATION OF AGAROSE PLATES

1. Place the template under the plate so the pattern is centered.
2. Cut the wells using the well cutter (provided in the kit) in a gentle punching motion. Remove the agarose plugs with a flat-edged toothpick or spatula.

B. PREPARATING THE STANDARDS (SERIAL DILUTION)

1. Label four microtest tubes: 1:2, 1:4, 1:8, and 1:16.
2. Using a micropipet, add 50 microliters of Buffer to each tube.
3. With a fresh pipet tip, add 50 microliters of "Standard" to the tube labeled 1:2. Mix.
4. With a fresh pipet tip, transfer 50 microliters of the 1:2 dilution to the tube labeled 1:4. Mix.
5. With a fresh pipet tip, transfer 50 microliters of the 1:4 dilution to the tube labeled 1:8. Mix.

6. With a fresh pipet tip, transfer 50 microliters of the 1:8 dilution to the tube labeled 1:16. Mix.
7. There are now five antigen samples for the standard curve (see chart).

DILUTION	CONCENTRATION
Undiluted	2 mg/ml
1:2	1 mg/ml
1:4	0,5 mg/ml
1:8	0,25 mg/ml
1:16	0,125 mg/ml

C. PRACTICE WELL LOADING (OPTIONAL)

This experiment contains practice loading solution. This solution is included to allow instructors and students to practice loading the sample wells before performing the actual experiment. Use a micropipetting device or one of the plastic transfer pipettes included in the experiment to practice loading the sample wells with the practice loading solution. Make enough copies of the template for each lab group.

1. One practice plate should be prepared for every two groups. Enough reagents have been provided for this purpose.
2. Using the well cutters provided, cut several wells in the agarose as shown in the template below. Refer to Student Instructions for preparation of sample wells.
3. Practice loading the sample wells with the Practice Loading Solution using a micropipetting device. Load 5 µl per well and make sure the sample covers the entire surface of the well. If a micropipetting device is not available, use the transfer pipets provided, taking care not to overfill the wells. If using transfer pipets, put in just enough sample to cover the bottom of the well.

D. LOADING THE SAMPLES

1. On the bottom of the plate, number the wells on the perimeter of the plate 1 through 5. Leave the center well unlabeled.
2. Load wells 1 through 5 using the same pipette tip or transfer pipette. In well #5, load 5 µl of the 1:16 antigen dilution. Make sure the sample covers the entire surface of the well by carefully spreading it with the pipette tip.
3. In well #4, load 5 µl of the 1:8 antigen dilution.
4. In well #3, load 5 µl of the 1:4 antigen dilution.
5. In well #2, load 5 µl of the 1:2 antigen dilution.
6. In well #1, load 5 µl of the undiluted antigen.

NOTE: You may use the same pipette tip or transfer pipette to load wells #1 through #5, starting with the most dilute antigen dilution and ending with the most concentrated. Use a fresh tip for the unknown.

7. With a fresh pipette tip or microtipped transfer pipette, load 5 µl of your unknown in the center well.
8. Label the cover of the Petri dish with your lab group number or your initials. Place the cover on the dish, place the dish right side up (do not invert) inside the incubation chamber on the paper toweling. Cover the incubation chamber and place in a 37°C incubation oven or at room temperature for 24 to 48 hours.

E. READING THE RESULTS

The precipitin rings will be visible in 24 to 48 hours. Carefully hold a plate up so that the overhead room lights shine through it. You should be able to see opaque circles around each well where antigen and antibody have precipitated.

With a ruler, measure the diameter (through the centers of the wells) of the precipitin ring in millimeters. To plot the standard curve, square the diameter value and plot antigen concentration on the X-axis and the diameter squared on the Y-axis. Draw the best fit line through these points. Calculate the value of the unknown antigen concentration from this graph.

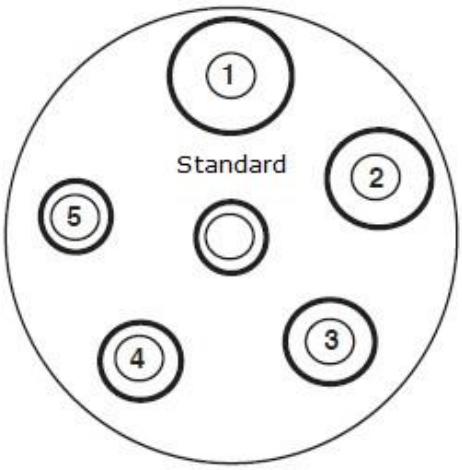
6. EXPERIMENTAL RESULTS

6.1 Experimental Results and Analysis

On regular graph paper, a linear standard curve should be obtained. If the curve is not linear, the unknown concentration cannot be accurately determined. Precipitin rings will vary based on the concentration of the antigens, antibody, agarose and the time and temperature of incubation.

From the standard curve, the unknown concentration can be determined by finding the diameter squared value on the Y-axis, finding the intersecting point on the standard curve line, and obtaining the value on the X-axis. The value on the X-axis is the concentration of antigen in the solution.

The concentration provided was **0.40 mg/ml**.



6.2 Study Questions

Answer the following study questions in your laboratory notebook or on a separate worksheet.

1. What do the circular precipitin rings represent?
2. Why do the ring sizes change until equilibrium is reached?
3. Predict the results if a very low concentration of antigen were loaded into a well. What would happen if not enough antibody was incorporated into the agarose?
4. Compare and contrast Radial Immunodiffusion with its close relative, the Ouchterlony plate technique.